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Hepatic lymphatic vascular system in health and disease

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Abstract

In recent years, significant advancements have been made in the study of lymphatic vessels with the identification of their specific markers and the development of research tools that have accelerated our understanding of their role in tissue homeostasis and disease pathogenesis in many organs. Compared to other organs, the lymphatic system in the liver is understudied despite its obvious importance for hepatic physiology and pathophysiology. This review article presents basic knowledge of the hepatic lymphatic system and its role in a range of liver-related pathological conditions such as portal hypertension, ascites formation, malignant tumors, liver transplantation, congenital liver diseases, non-alcoholic fatty liver disease (NAFLD), and hepatic encephalopathy. The article concludes with a discussion on the modulation of lymphangiogenesis as a potential therapeutic strategy for liver diseases.

Keywords

Lymphangiogenesis; VEGFs; liver fibrosis; liver transplantation; portal hypertension

Introduction

The liver is recognized as the largest lymph producing organ, accounting for nearly 25 – 50% of lymph passing through the thoracic duct[1–3]. Their functional importance has been discussed mainly in regard to the regulation of lymph production, which is highly influenced by hemodynamic changes in the intrahepatic microcirculation. While lymphatic vessel numbers are known to increase (via lymphangiogenesis, new lymphatic vessel formation) in many liver diseases, the mechanism of lymphangiogenesis in the liver remains poorly

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understood[4, 5]. In addition to the maintenance of fluid homeostasis, hepatic lymphatic vessels are highly involved in the immune system and lipid metabolism by transporting various immune cells, antigens and lipids to lymph nodes. Despite these roles in key processes in the liver, hepatic lymphatic vessels have been understudied.

In the last decade, significant advancements have been made in the study of lymphatic vessels, including the identification of specific markers and the development of research tools, accelerating our understanding of their roles in tissue homeostasis and disease pathogenesis in many organs[6–8]. Lymphatic vessels exhibit a variety of immunoregulatory functions by expressing a wide range of chemokines and receptors. Thus, lymphatic vessels are more than conduits removing lymph, immune cells and cellular products from organs and tissues.

This review article aims to provide fundamental knowledge of the hepatic lymphatic system and its implications in liver diseases. We will discuss: 1) the fundamental knowledge of liver lymphatics, including structure, markers, lymph and lymphatic drainage; 2) lymphatics and the immune system; 3) mediators and signals leading to hepatic lymphangiogenesis; 4) lymphatics in liver diseases and their complications, including portal hypertension, ascites, malignant tumors, liver transplantation, congenital liver diseases, non-alcoholic fatty liver disease (NAFLD), and hepatic encephalopathy; 5) therapeutic potential and future directions in the study of liver lymphatics. For details of the biology of the hepatic lymphatic system, other review papers are referenced in Supplementary Table 1.

1. Hepatic lymphatic system

1–1. Structure of lymphatics (Figure 1)

A history of studies of the hepatic lymphatic system and how hepatic lymph reaches hepatic lymphatic vessels are described in an excellent review paper by Ohtani & Ohtani[5]. The lymphatic vascular system consists of lymphatic capillaries (also known as initial lymphatics) and collecting lymphatic vessels. Lymphatic capillaries consist of a single layer of lymphatic endothelial cells (LECs) with no coverage of “lymphatic muscle” cells. In contrast to lymphatic capillaries, collecting lymphatic vessels are covered with lymphatic muscle cells and located downstream of lymphatic capillaries. “Lymphatic vessels” are often defined as an intermediate structure between lymphatic capillaries and lymphatic collecting vessels. In this review article, however, we use the term “lymphatic vessels” to describe both lymphatic vessels and lymphatic capillaries, because lymphatic capillaries are most common in the liver. Endothelial cells of lymphatic capillaries have discontinuous, “button-like” junctions (buttons), which are strikingly different from continuous, “zipper-like” junctions (zippers) of collecting lymphatic vessels and blood vessels[9, 10]. The button-like junctions are thought to allow efficient entry of fluid, antigens and immune cells into lymphatic capillaries (Figure 2). In pathological conditions, it is reported that lymphatic capillaries lose this “button-like” structure with a change to the less permeable “zipper-like” structure[11, 12]. This change of junctional structure could impair transport of fluid and substances to lymphatic capillaries, thereby decreasing their clearance from tissues.

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1–2. Markers of lymphatics

Markers of LECs include vascular endothelial growth factor receptor 3 (VEGFR3)[13, 14], lymphatic vessel endothelial hyaluronan receptor 1 (Lyve1)[15, 16], prospero homeobox protein 1 (Prox1)[17] and podoplanin (also known as D2–40)[18]. However, these LEC markers are also expressed in other liver cells, which has made studies of the hepatic lymphatic system challenging[4, 19]. For example, VEGFR3 and Lyve1 are expressed in liver sinusoidal endothelial cells (LSECs), while Prox1 is expressed in hepatocytes. In the normal liver, the high localization of lymphatic capillaries in the portal tract allows these markers to be used for their identification. Because lymphatic capillaries are not covered by αSMA-positive smooth muscle cells, αSMA-labeling can differentiate lymphatics from blood vessels in the portal tract. In human liver specimens, podoplanin/D2–40 has frequently been used for identification of lymphatic vessels. However, identification of unique LEC proteins that are not expressed in other liver cells is indispensable for advancement of our understanding of lymphatic vessels in the liver.

The presence of LECs in the liver tends to be overlooked at least in part because they only account for a very small portion of the liver EC population in the normal liver, while liver cirrhosis increases the contribution of LECs to total ECs by 20-fold[20]. A recent study of the liver EC population using single-cell RNA sequencing (scRNA-seq) analysis identified several novel genes highly expressed in LECs, but with absent or very minimal expression in LSECs and arterial/venous ECs[20]. These potential liver LEC markers include Mmrn1, Rassf9, Tbx1 and interleukin 7 (IL7). Characterization of these genes may help to understand unique functions of LECs as opposed to LSECs.

1–3. Lymph and lymphatic drainage

Hepatic lymph (lymphatic fluid) consists of sinusoidal plasma filtered into the space of Disse[21], an interstitial space between LSECs and hepatocytes, through fenestrae of LSECs. Although detailed hepatic lymph contents have not yet been specified, these contents potentially include cellular byproducts discharged from hepatic cells in the space of Disse (Figure 1). Lymph is then thought to flow into lymphatic vessels located in three regions: portal, hepatic venous and sub-capsular areas[22]. Among them, lymphatic vessels in the portal tract are considered to be the primary site of hepatic lymph drainage, covering around 80% of the lymph produced by the liver[23]. Because the hepatic sinusoids are highly permeable and oncotic pressure along the sinusoids is negligible, fluid in the hepatic sinusoids can flow through the channels traversing the limiting plate to the interstitial space of the portal tract, following hydrostatic pressure gradients.

Lymphatic capillaries in the portal tract coalesce into collecting lymphatic vessels surrounded by lymphatic muscle cells outside the liver[4, 5, 24]. Lymphatic muscle cells covering collecting lymphatic vessels help to pump lymphatic fluid into regional lymph nodes known as hepatic or hilar lymph nodes located in the hepatic hilum. From these lymph nodes, lymphatic fluid flows to celiac lymph nodes through collecting lymphatic vessels and then drains to the cisterna chyli located at the lower end of the thoracic duct. Lymphatic capillaries running along the hepatic vein merge into 5–6 large lymphatic vessels, which traverse along with the inferior vena cava toward posterior mediastinal lymph nodes

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through the diaphragm. Lymphatic vessels along the hepatic capsule drain lymphatic fluid underneath the capsule of the convex surface of the liver to regional lymph nodes such as diaphragmatic lymph nodes in the thoracic region and then to mediastinal lymph nodes, similar to those along the hepatic vein.

While studies have suggested specific draining lymph nodes associated with lymphatic vessels in respective areas as mentioned above, the route of lymphatic drainage may be more complex and further analysis may be needed. Lymphatic vessels along the portal tract and the hepatic vein are called “the deep lymphatic system”, while those along the hepatic capsule are called “the superficial lymphatic system”[5]. A recent study[25] that examined drainage patterns of the deep lymphatic system in the mouse liver by lymphangiography showed that hepatic lymphatic fluid was preferentially drained into regional hilar lymph nodes when it came from the right or left lobe. However, hepatic lymphatic fluid from the median lobe was mainly drained to mediastinal lymph nodes rather than hilar lymph nodes. These observations may suggest that the hepatic lymphatic drainage system is organized in a lobe-specific manner in mice.

2. Lymphangiogenesis

2–1. Pro- and anti-lymphangiogenic factors in the liver

In adults, lymphatic vessels generally remain quiescent in normal conditions, and lymphangiogenesis occurs in pathological conditions such as tissue repair, inflammation and tumor-related conditions. Many cytokines and growth factors have been reported to promote or inhibit lymphangiogenesis in other organs. Among them, those cytokines and factors detected in the liver in physiological and pathophysiological conditions (thus not necessarily studied for lymphangiogenesis) are listed in Supplementary Table 2 as potential pro-and anti-lymphangiogenic factors in the liver. Cellular sources of these factors have not been fully identified in the liver.

2–2. VEGF-C/D and VEGFR3 signaling

Signals mediated by members of the VEGF and VEGFR families are known to play central roles in angiogenesis and lymphangiogenesis (Figure 3)[26–28]. VEGF-A binds to VEGFR1/Flt1 and VEGFR2/KDR[29, 30] and mediates angiogenesis, while VEGF-B and placental growth factor (PIGF) bind only to VEGFR1/Flt1[27, 31]. VEGF-C and D bind strongly to VEGFR3/Flt4, leading to lymphangiogenesis, while they also bind very weakly to VEGFR2/KDR[27, 32]. VEGF-C and D are initially synthesized as precursor forms that subsequently undergo proteolytic cleavage, removing their C and N-terminal propeptides for induction of lymphangiogenesis[27]. Currently, five different proteases are known to cleave VEGF-C, including plasmin, ADAMTS3 (A Distintegrin and Metalloprotease with Thrombospondin Motifs-3), prostate-specific antigen, cathepsin D and thrombin. All these proteases except for ADAMTS3 can also activate VEGF-D. The usual VEGF-C cleaving enzyme is ADAMTS3, which requires binding to its cofactor, CCBE1 (collagen and calcium binding EGF domains 1), for successful pro-VEGF-C activation[33, 34]. The inability of ADAMTS3-CCBE1 to activate VEGF-D[33] may suggest that biological events induced by VEGF-C and D are tissue or context dependent.

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3. Lymphatics and the immune system

LECs express various chemokines and recruit immune cells. The most-studied chemokine in this regard is C-C motif chemokine ligand 21 (CCL21), a lymphoid homing chemokine[35]. CCL21 guides dendritic cells (DCs), which express its receptor C-C chemokine receptor type 7 (CCR7), and other CCR7-expressing immune cells such as T-cell subsets (naïve, memory and T regulatory T cells) and neutrophils to lymph nodes through lymphatic vessels[36–38]. LECs also express intracellular adhesion molecule 1 (ICAM1)[39] as well as C-X3-C motif chemokine ligand 1 (CX3CL1) to guide DCs to lymphatic vessels in inflamed skin[40]. Further, LEC-derived sphingosine-1-phosphate (S1P) has been identified as a critical lipid mediator molecule that interacts with S1P receptor 1 (S1P1) on T cells to promote their egression from lymph nodes[41] and spleen[42].

Besides T cell migration, LECs also regulate T cell function. Human and murine LECs express major histocompatibility complex (MHC) class I and class II molecules and may directly induce T cell tolerance, which prevents self-activation of T cells to innocuous proteins and self-antigens in normal conditions[43–46]. Further, multiple peripheral tissue antigens (PTAs) expressed in LECs are known to play an important role in inducing T cell tolerance and mediate deletion of self-reactive CD8+ T cells. LECs also secrete immunoregulatory factors, including TGF-β, indoleamine-2,3-dioxygenase (IDO) and nitric oxide, to suppress T cell activation[46, 47].

These observations of LEC's immunomodulatory function from other organs could be applicable to hepatic LECs and help to understand the role of lymphatic vessels in liver functions. In chronic liver diseases, lymphatics play a role in immune cell trafficking. An increase in the number of CCL21-expressing LECs has been reported in the livers of patients with non-alcoholic steatohepatitis (NASH)[48]. Although the relationship between increased levels of CCL21 and hepatic lymphatics has not been investigated, other studies also reported an increase in hepatic CCL21 expression in primary biliary cholangitis (PBC), primary sclerosing cholangitis (PSC) and alcohol-associated liver disease (ALD)[49, 50]. Similarly, S1P signaling was shown as one of the key players in metabolic diseases and various liver pathologies including NAFLD, NASH and liver fibrosis[51]. The underlying mechanisms have been explored in regard to S1P's role in hepatocytes and hepatic stellate cells in their regulation of hepatic glucose and lipid metabolism[52]. It is interesting to examine the role of S1P signaling in these diseases through its effect on hepatic lymphatics.

4. Lymphatics in liver diseases

4–1. Hepatic lymphangiogenesis

Lymphangiogenesis with increased and enlarged lymphatic vessels was reported in fibrotic/cirrhotic rat livers induced by carbon tetrachloride (CCl4)[53] as well as in patients with chronic viral hepatitis/cirrhosis[54]. In two rat models of portal hypertension (portacaval shunt and partial portal vein ligation), upregulation of VEGFR3 expression was observed. This observation leads us to speculate the occurrence of lymphangiogenesis in these models of portal hypertension as well[55]. In addition, liver specimens from patients with early-stage PBC showed an increase in the number and the luminal area of lymphatic

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vessels, indicating that lymphangiogenesis occurred even at the early-stage of PBC[56]. Further, microarray analysis revealed a 4-fold increase in VEGF-D expression in endothelial cells isolated from CCl_4-induced cirrhotic rat livers compared with control rat livers[57], suggesting the role of VEGF-D in hepatic lymphangiogenesis observed in CCl_4-induced cirrhotic rat livers.

4–2. Hepatic lymph production in liver cirrhosis with portal hypertension

Resistance to sinusoidal blood flow increases in cirrhotic livers because of their architectural deformations due to massive fibrosis. Consequently, hydrostatic pressure in the sinusoids is elevated, and plasma components filtrated through the sinusoids (which form lymph) increase[4, 58]. This mechanism is supported by observations in which lowering portal venous pressure by portocaval shunt surgery decreased the lymph flow rate in cirrhotic dogs[59] and patients[60, 61]. Transjugular intrahepatic portosystemic shunts (TIPSs) were also shown to have the same effect on thoracic duct lymph flow[62]. In cirrhosis, hepatic lymph production significantly increases in both humans[60] and rats[63, 64]. It was reported that patients with liver cirrhosis showed three to six times higher lymph flow rates in the thoracic duct than those patients without cirrhosis[60]. In this study, the thoracic duct was cannulated to measure the flow rate and compositions of lymph. For cirrhotic rats with portal hypertension, one study reported that hepatic lymph flow (measured by cannulating the hepatic lymph trunk) increased nearly 30-fold compared to control rats[63], while another study with cirrhotic rats estimated a 6-fold increase using a similar method of measurements[64]. This discrepancy in the magnitude of flow could be due to the timing of measurements or the severity of cirrhosis. In cirrhotic patients, the diameter of the thoracic duct was also dilated by two to four times, and the lymphatic flow rate of the thoracic duct increased by three to six times[60]. Increased collateral lymph flow in the mediastinum and esophagus results in bloody lymph in the thoracic duct due to communication opened between lymphatic vessels and veins[65].

4–3. Ascites formation

Ascites formation in association with cirrhosis is one of the most recognized clinical manifestations of disorders of the lymphatic system[58]. Currently, the most accepted theory of ascites formation is the “forward theory” (Figure 4)[66–68]. According to this theory, splanchnic arterial vasodilation caused by portal hypertension results in underfilling of the splanchnic arterial circulation or hypovolemia. In moderate stages, hypovolemia is compensated by renal retention of sodium and water[69]. However, severe portal hypertension and splanchnic arterial vasodilation make sodium and water retention persistent and lead to leaking fluid into the peritoneal cavity. In cirrhosis, decreased oncotic pressure caused by hypoalbuminemia also promotes fluid leakage into the peritoneal cavity[70]. As described above, increased intrahepatic vascular resistance due to liver cirrhosis results in elevated hydrostatic pressure and the consequent increase in hepatic lymph production as well.

Levitt *et al.* looked into the local mechanism of ascites formation in the peritoneal cavity and developed a quantitative model[71]. Equations they formulated described the fluid flux based on hydrostatic pressure and colloid osmotic pressure, and were validated by actual

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parameters obtained from animal experiments and clinical data. This model demonstrates that an elevation of portal pressure itself does not cause appreciable ascites. Ascitic fluid starts to accumulate when capsular or superficial lymphatics of the liver rupture and hepatic lymph with a high protein concentration leaks into the peritoneal cavity. This ascitic fluid with high colloidal pressure causes transport of water from the mesentery to the peritoneal cavity, which results in further increases of ascitic fluid. The lymph leakage from the surface of the liver has been observed directly by laparoscopy and is known as the so-called ‘weeping liver’[72, 73], and no studies have yet quantified this lymph leakage. It is thought that ascites forms when the total fluid flux into the peritoneal cavity exceeds the lymph draining capacity of the peritoneum, which is approximately 50 ml/hr at most[74–77].

4–4. Malignant tumors

Lymphatic vessels play a pivotal role in the pathogenesis of malignant tumors as a pathway through which tumor cells spread[78, 79]. The incidence of lymph node metastasis differs among malignant tumors. In liver cancer, lymph node metastasis was observed in 5.1% of hepatocellular carcinoma (HCC) and 45.1% of intrahepatic cholangiocarcinoma (ICC)[80]. The prognosis of tumor-bearing patients with lymph node metastasis is worse than the cases without lymph node metastasis. In ICC, the lymphatic vessel density of surgically resected ICCs increased as the extent of malignancy worsened [81] and was correlated with higher incidences of lymphatic metastasis[82].

As mentioned above, CCL21 in LECs recruits CCR7-expressing DCs toward lymphatic vessels and facilitates their egression from the liver[83]. However, CCR7 is also expressed by a variety of malignant tumors, and the CCL21–CCR7 axis is considered as one of the causal factors for lymph node metastasis[84–89]. A positive correlation between expression levels of CCR7 and lymph node metastasis was reported in HCC patients[90].

In addition, many malignant tumors are known to secrete lymphangiogenic factors such as VEGF-C and VEGF-D and promote lymphangiogenesis in adjacent tissues, which helps tumor cells to metastasize to lymph nodes[91]. Many studies have demonstrated that tumor-associated macrophages play a vital role in lymphangiogenesis in malignant tumors by secreting VEGF-C and VEGF-D[92–95]. In ICC, VEGF-C expression was associated with a higher rate of lymph node metastasis and poorer prognosis[81, 96]. In HCC, VEGF-C expression was shown to correlate positively with the size of tumors and the number of extrahepatic metastasis and negatively with disease-free survival time[97]. Plasma VEGF-C levels in patients with liver transplantation for HCC treatment were negatively associated with both their disease-free survival rates and overall survival rates[98].

A recent study demonstrated that ICC-derived platelet-derived growth factor D (PDGF-D) recruited and activated cancer-associated fibroblasts (CAFs) in stromal components adjacent to ICC. Activated CAFs then secreted VEGF-A and VEGF-C, inducing lymphangiogenesis and thereby promoting lymph node metastasis[99]. The study also showed that a VEGFR3 antagonist reduced tumor-associated lymphangiogenesis in a xenograft model using SCID (severe combined immunodeficiency) mice transplanted with PDGF-D secreting human ICC cell lines. No selective agents that specifically suppress lymphangiogenesis have been approved for clinical use[100]. A phase I clinical trial of VEGF-C neutralizing antibody

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VGX-100 for adult patients with advanced or metastatic solid tumors (NCT01514123) was finished in 2014, but a phase II trial has not been opened yet[101, 102]. A phase I clinical trial of anti-VEGFR3 monoclonal antibody LY3022856/IMC-3C5 for patients with advanced solid tumors demonstrated a minimal anti-tumor effect[103].

Contrarily, interesting findings that VEGF-C/VEGFR3 driven lymphangiogenesis enhances an anti-tumor effect of immunotherapy have recently been reported in some tumors. One study performed inoculation of a melanoma cell line into VEGF-C overexpressing mice intradermally to evaluate a synergistic effect of a VEGFR3 blocking antibody on adaptive immunotherapy[104]. Contrary to expectations, blocking VEGFR3 suppressed the effect of immunotherapy and worsened the survival rate. It was demonstrated that VEGF-C overexpression facilitated intratumoral lymphangiogenesis, upregulated intratumoral CCL21 expression and promoted infiltration of CCR7-expressing naïve T cells into tumors. Conversely, VEGFR3 blocking inhibited intratumoral lymphangiogenesis, decreased intratumoral CCL21 expression and suppressed the number of intratumoral naïve T cells, which may have contributed to decreased sensitivity to immunotherapy. It was also shown that serum VEGF-C concentrations positively correlated with both T-cell activation after peptide vaccination and overall survival rates after a checkpoint blockade therapy in metastatic melanoma patients[104]. Another study using a mouse model of glioblastoma demonstrated that adeno-associated virus (AAV)-mediated VEGF-C gene transfer promoted meningeal lymphatic drainage and improved the survival rate[105]. Ligation of deep cervical lymph nodes, to which cerebrospinal fluid is drained through meningeal lymphatic vessels, as well as depletion of CD4- or CD8-positive T cells negated the beneficial effect of VEGF-C, indicating that the anti-tumor effect of VEGF-C was mediated by T cells that migrated to lymph nodes. It was also shown that VEGF-C gene transfer enhanced the effect of immunotherapy by anti-PD-L1 antibody. These findings indicate complex roles of tumor-associated lymphatic vessels, which act not only as a pathway for tumor dissemination, but also as an integral part of T-cell mediated anti-tumor immunity.

4–5. Liver transplantation

Graft rejection is one of the most serious concerns in solid organ transplantation. Alloimmunity (responses to non-self antigens from the same species) is established once alloantigens of the graft are drained into secondary lymphoid organs through lymphatic vessels and encounter T lymphocytes[106]. Therefore, the potential role of lymphangiogenesis in graft rejection has received considerable attention[4].

Post-transplant lymphangiogenesis in grafts was associated with acute cellular graft rejection in various organs (kidney[107], heart[108] and lung[109]) in humans. However, in a rat model of liver transplantation, increased post-transplant lymphangiogenesis in grafts was associated with long-term survival of recipients for more than 90 days. In addition, rats that had failed to graft by 11 days showed disappearance of lymphatic vessels from severely rejected areas. These observations may suggest that lymphangiogenesis plays a beneficial role in mitigation of inflammation at least in the early stage of liver transplantation[110]. This difference between the liver and other organs might be attributable in part to hepatic immune tolerance[111].

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Hepatic immune tolerance was first reported in 1969, describing that liver allotransplantation did not lead to rejection of the second allograft transplanted from the same donor[112]. In liver transplantation, human leukocyte antigen (HLA) mismatch is not a problem[113], and immunosuppressant requirements are more mild compared with other organ transplantations[114]. While the mechanism of hepatic immune tolerance is not fully understood, unique phenotypes of liver resident cells may play a role. For example, it was reported that Kupffer cells suppressed T cell proliferation through secretion of nitric oxide in response to interferon-γ[115] and that LSECs negatively regulated activated T cells via expression of LSEC-specific lectin that recognizes these activated T cells[116]. In addition, different responses of hepatic DCs compared to other DCs may contribute to immune tolerant environments in the liver. One study demonstrated that hepatic DCs secreted anti-inflammatory cytokine IL10 upon toll-like receptor (TLR) 4 stimulation, while peripheral DCs secreted multiple proinflammatory cytokines[117]. This study also showed that CD4-positive T cells initially stimulated by hepatic DCs were less responsive to restimulation than those T cells pre-stimulated by blood DCs. Another study demonstrated that hepatic DCs secreted less inflammatory cytokine IL12 than spleen DCs upon activation by TLR9 ligand[118]. This study also showed that T cells stimulated by hepatic DCs secreted less IFN-γ than those stimulated by spleen DCs. Given that DCs present antigens to T cells in lymph nodes to establish alloimmunity in organ transplantation, hepatic lymphangiogenesis might contribute to immune tolerance in the liver by sending tolerant hepatic DCs to lymph nodes.

4–6. Congenital liver diseases

Lymphedema cholestasis syndrome (LCS) is an autosomal recessive syndrome characterized by primary lymphedema and cholestasis in the neonatal period with intermittent recurrences in childhood. A Norwegian type of LCS (a.k.a., Aagenaes syndrome) has the same haplotype on chromosome 15q and is classified as LCS1[119]. Out of 40 LCS1 patients studied, six patients developed severe cirrhosis with death or liver transplantation in infancy or early childhood, and three patients slowly developed progressive cirrhosis[120]. Recently, a mutation of CCBE1, a secreted protein essential for VEGF-C activation, was reported to be responsible for one LCS patient without the LCS1 mutation[121]. In addition, the CCBE1 mutation was identified in another patient who developed recurrent cholangitis at age 52 with a family history of primary lymphedema in lower limbs[122]. While the mechanism of cholestasis in LCS remains to be elucidated, these clinical findings may indicate that malfunctions of the hepatic lymphatic system are involved in the pathogenesis.

4–7. Non-alcoholic fatty liver disease (NAFLD)

Increased lymphatic vessel density was observed in areas of fibrosis and immune cell infiltration in patients with chronic liver diseases, including NASH[48]. In this study, scRNA-seq analysis of LECs from healthy controls and NASH patients revealed upregulation of genes related to IL13 signaling[48], which has been shown to regulate lymphangiogenesis negatively by inhibiting Prox1 expression as well as migration and proliferation of LECs[123, 124]. This study also showed in a preclinical model of NASH that oxidized low-density lipoprotein (oxLDL), which is known to be elevated in NASH livers[125, 126], could induce IL13 upregulation and reduce Prox1 transcript levels and

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LEC identity, suggesting an oxLDL-mediated mechanism of increased IL13 expression and LEC identity changes in NASH[48]. Later, this group recapitulated these findings in mice fed a high-fat, high cholesterol diet (another NASH model)[127]. They also showed that lymphatic transport activity was impaired in NASH mice, but it was rescued by administration of recombinant vascular endothelial growth factor C (rVEGF-C) concomitant with amelioration of hepatic inflammation. Drugs with pro-lymphangiogenic properties could be a new therapeutic strategy for NASH.

In recent years, a growing body of evidence has indicated that impairment of lipid transport by the lymphatic system (i.e., lacreals) could have systemic metabolic consequences[128–131]. Similarly, many studies have addressed the relationship between lymphatic dysfunction and obesity. For example, in heterozygous mice lacking Prox1 ($Prox1^{+/-}$), lymphatic vascular defects and adult-onset obesity with elevated triglycerides were observed[131]. Lymphatic-specific Prox1 restoration rescued the adult-onset obesity phenotype in $Prox1^{+/-}$ mice, directly linking lymphatic dysfunction to the development of obesity. Obesity could also facilitate lymphatic dysfunction. Multiple mouse models of obesity demonstrated impaired lymphatic functions characterized by leaky lymphatics and reduced pumping capacities of collecting lymphatic vessels[128, 132–136]. Thus, it may be interesting to investigate NAFLD with a focus on impairment of lipid transfer due to lymphatic dysfunction.

4–8. Hepatic encephalopathy

Hepatic encephalopathy (HE) is a serious neurologic complication in patients with severe liver dysfunction resulting from acute liver failure or decompensated cirrhosis. In 2015, two groups of investigators re-discovered the presence of meningeal lymphatic vessels located underneath the skull[137, 138] and revealed that they are the major route for discharging waste materials and immune cells from the brain to lymph nodes in the neck called the deep cervical lymph nodes. Because of its critical functions for brain homeostasis, the meningeal lymphatic system has been receiving great attention as a potential therapeutic target for neurodegenerative and neuroinflammatory disorders.

A recent study using rats with 4-week bile duct ligation provided insight into the role of the meningeal lymphatic system in HE[139]. The study demonstrated that overexpression of VEGF-C via adeno-associated virus 8 (AAV8)-VEGF-C injection to the cisterna magna ameliorated HE, including motor dysfunction, by decreasing neuroinflammation and microglia activation through increased meningeal lymphangiogenesis and thereby enhanced meningeal drainage to the cervical lymph nodes. Manipulation of meningeal lymphangiogenesis could be a novel therapeutic strategy for HE.

5. Therapeutic potential and future directions

An increasing body of evidence supports an idea that modulation of lymphangiogenesis or lymphatic drainage is an effective therapeutic strategy for a wide range of pathological conditions[79, 140], including rheumatoid arthritis[141], psoriasis-like chronic dermatitis[142], inflammatory bowel disease[143], interstitial nephritis[144] and glioblastoma[105]. For liver diseases, several preclinical studies have shown

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that administration of VEGF-C or D ameliorates disease conditions by increasing lymphangiogenesis and lymphatic drainage. On the other hand, blocking lymphangiogenesis could also be an appropriate option for diseases such as cholangiocarcinoma where tumor-induced lymphangiogenesis may promote metastasis of malignant tumors.

An understanding of detailed molecular and cellular mechanisms of hepatic lymphangiogenesis is essential for its modulation. It is not adequately known which cells produce pro- or anti-lymphangiogenic factors and regulate hepatic lymphangiogenesis. Macrophages have been recognized for regulation of hepatic lymphangiogenesis by producing lymphangiogenic factors such as VEGF-C and D at the site of lymphatic vessel expansion[145]. Cholangiocarcinoma cells produce PDGF-D, which induces VEGF-C expression in myofibroblasts in tumor microenvironments, facilitating lymphangiogenesis and subsequent metastasis[99]. These observations may indicate that distinct mechanisms of lymphangiogenesis exist in different liver diseases.

While blocking VEGF-C is effective for decreasing lymphatic vessels and may prevent tumor metastasis, it will also reduce recruitment of T-cells for their tumor-killing activity[105]. Thus, identification of alternative lymphangiogenic pathways other than the VEGF-C/D/VEGFR3 axis, including additional pro- and anti-lymphangiogenic factors, will likely increase novel therapeutic options for cholangiocarcinoma in particular and for liver diseases in general[4]. A recent study showed thrombospondin (THBS)1, THBS2 and pigment epithelial-derived factor (PEDF) from ICC in intrahepatic tumor microenvironments promoted cancer-associated lymhangiogenesis, suggesting that THBS1, THBS2 and PEDF could be promising targets to reduce cancer-associated lymphangiogenesis and counteract invasiveness of ICC[146].

An origin of LECs for hepatic lymphangiogenesis in pathological conditions remains to be identified. A previous study reported a very small contribution of bone marrow-derived hematopoietic stem cells to LECs in the regenerating mouse liver[147]. Further studies with lineage tracing analysis are warranted to identify the origin of LECs in hepatic lymphangiogenesis.

Finally, it can be expected that hepatic lymph contains 80 to 90% of the proteins present in plasma[1, 3]. The content of the lymph, including self-peptides derived from intracellular, membrane-associated or matrix proteins, has been gaining attention as it may represent local conditions more accurately than blood[148, 149], suggesting the potential for containing more useful biomarkers. Moreover, lymph carries apoptotic cellular materials, cytokines, cell-derived microparticles and infectious agents, mediating communications between lymph generating organs (e.g., liver) and their associated draining lymph nodes, which is critical for host defense[149]. The relevance of small molecules and vesicles discharged via lymphatics to the etiology of liver diseases remains to be explored.

In conclusion, the lymphatic system in the liver remains underexplored. Given its pivotal homeostatic roles in the liver and other organs, investigations in this area will advance our knowledge of liver physiology and pathophysiology and lead to the development of effective therapies for liver diseases.

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Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of abbreviations

NAFLD	non-alcoholic fatty liver disease
LECs	lymphatic endothelial cells
VEGFR3	vascular endothelial growth factor receptor 3
Lyve1	lymphatic vessel endothelial hyaluronan receptor 1
Prox1	prospero homeobox protein 1
LSEC	liver sinusoidal endothelial cell
ECs	endothelial cells
IL7	interleukin 7
PlGF	placental growth factor
ADAMTS3	A Distintegrin and Metalloprotease with Thrombospondin Motifs-3
CCBE1	collagen and calcium binding EGF domains 1
DCs	dendritic cells
CCL21	C-C motif chemokine ligand 21
CCR7	C-C chemokine receptor type 7
ICAM1	intracellular adhesion molecule 1
CX3CL1	C-X3-C motif chemokine ligand 1
S1P	sphingosine-1-phosphate
S1P1	S1P receptor 1
MHC	histocompatibility complex
PTAs	peripheral tissue antigens
IDO	indoleamine-2,3-dioxygenase

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NASH	non-alcoholic steatohepatitis
PBC	primary biliary cholangitis
PSC	primary sclerosing cholangitis
ALD	alcohol-associated liver disease
CCl4	carbon tetrachloride
TIPS	Transjugular intrahepatic portosystemic shunt
HCC	hepatocellular carcinoma
ICC	intrahepatic cholangiocarcinoma
AAV	adeno-associated virus
PDGF-D	platelet-derived growth factor D
CAFs	cancer-associated fibroblasts
HLA	human leukocyte antigen
TLR4	toll-like receptor 4
LSC	lymphedema cholestasis syndrome
HE	hepatic encephalopathy
oxLDL	oxidized low-density lipoprotein
PEDF	pigment epithelial-derived factor
THBS1	thrombospondin 1

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Key points

- The liver is recognized as the largest lymph producing organ.
- Hepatic lymphatic vessels are involved in fluid homeostasis, the immune system and lipid metabolism by transporting various immune cells, antigens and lipids to lymph nodes.
- While lymphatic vessel numbers are known to increase (via lymphangiogenesis) in many liver diseases, the mechanism of lymphangiogenesis in the liver remains poorly understood.
- VEGF-C and D bind to VEGFR3/Flt4, leading to lymphangiogenesis, while VEGF-A binds to VEGFR1/Flt1 and VEGFR2/KDR, mediating angiogenesis.
- Ascites formation in association with cirrhosis is one of the most recognized clinical manifestations of disorders of the lymphatic system.
- While lymphatics help to reduce inflammation, they also serve as routes of cancer metastasis.
- Modulation of lymphangiogenesis or lymphatic drainage could be an effective therapeutic strategy for a wide range of pathological conditions, including liver diseases.

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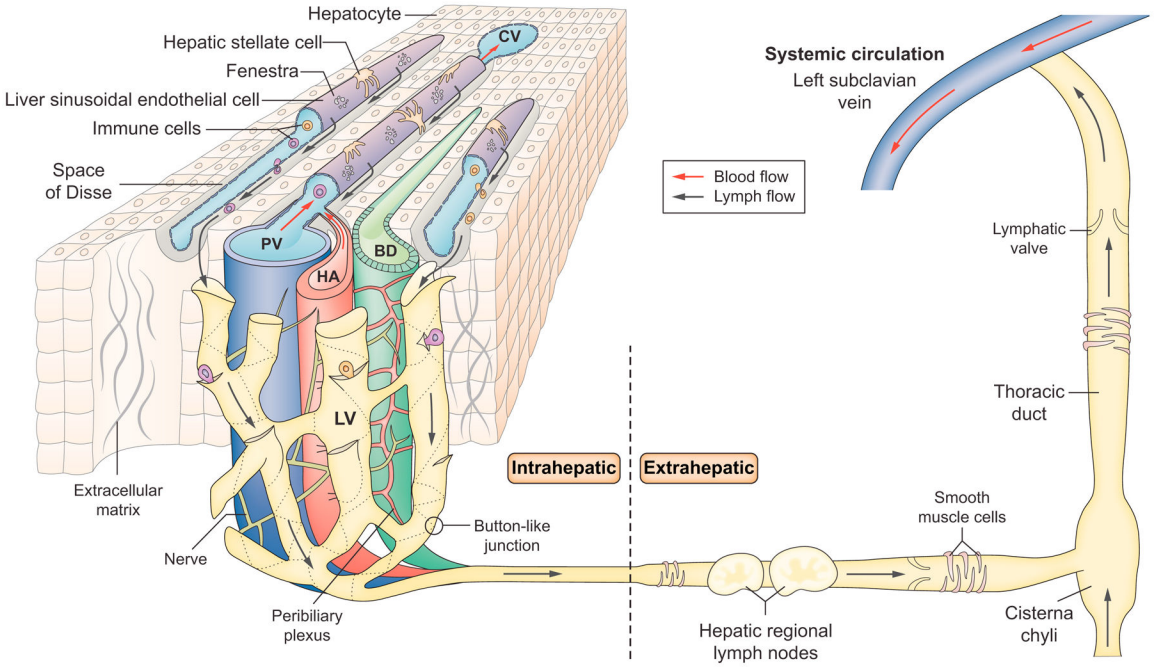

Figure 1. Hepatic lymphatic system

Lymph is thought to flow into lymphatic vessels located in three regions: portal, hepatic venous and sub-capsular areas. The illustration shows lymphatic vessels in the portal tract, the primary site of hepatic lymph drainage covering around 80% of the lymph produced by the liver. Because the hepatic sinusoids are highly permeable due to fenestrae, fluid in the hepatic sinusoids can flow through the channels traversing the limiting plate to the interstitial space of the portal tract. Endothelial cells of lymphatic capillaries have discontinuous, “button-like” junctions, which allow efficient entry of fluid, antigens and immune cells into lymphatic capillaries. Lymphatic capillaries in the portal tract coalesce into collecting lymphatic vessels surrounded by lymphatic muscle cells outside the liver. Lymphatic muscle cells covering collecting lymphatic vessels help to pump lymph into regional lymph nodes located in the hepatic hilum and then to the cisterna chyli located at the lower end of the thoracic duct. Lymph finally drains into the left subclavian vein via the thoracic duct and returns to the systemic blood circulation. LV: Lymphatic vessel, PV: Portal vein, HA: Hepatic artery, BD: Bile duct, CV: Central vein, ECM: Extracellular matrix protein, HSC: Hepatic stellate cell

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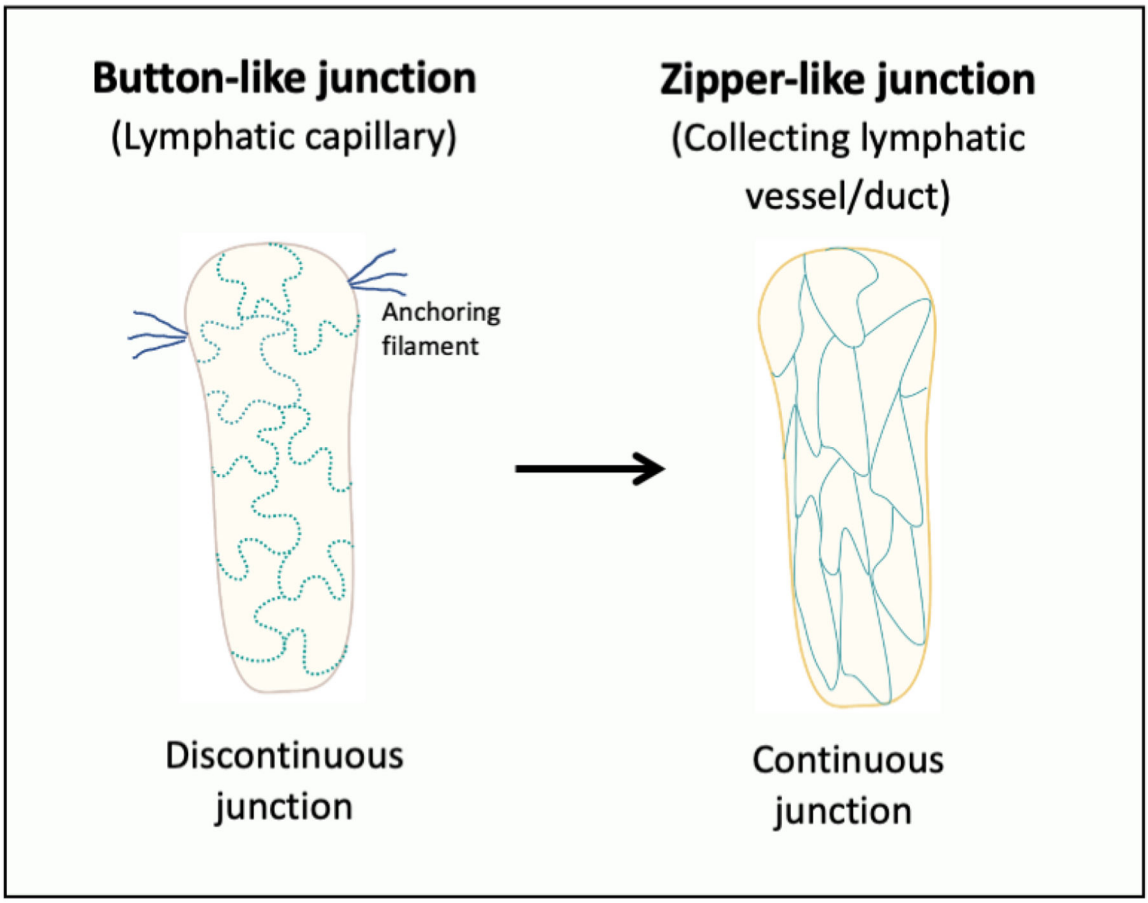

Figure 2. Button vs. Zipper-like structures

The lymphatic vascular system includes lymphatic capillaries (also known as initial lymphatics) and collecting lymphatic vessels. Lymphatic capillaries consist of a single layer of lymphatic endothelial cells. Endothelial cells of lymphatic capillaries are attached by anchoring filaments to surrounding extracellular matrix proteins, which support their vessel structure. Endothelial cells of lymphatic capillaries have discontinuous, “button-like” junctions, which allow efficient entry of fluid, antigens and immune cells into lymphatic capillaries. In contrast, collecting lymphatic vessels have “zipper-like” junctions, similar to blood vessels. In pathological conditions, lymphatic capillaries lose this “button-like” structure with a change to the less permeable “zipper-like” structure, which results in impaired transport of fluid and substances to lymphatic capillaries, thereby decreasing their clearance from tissues. Created with BioRender.com.

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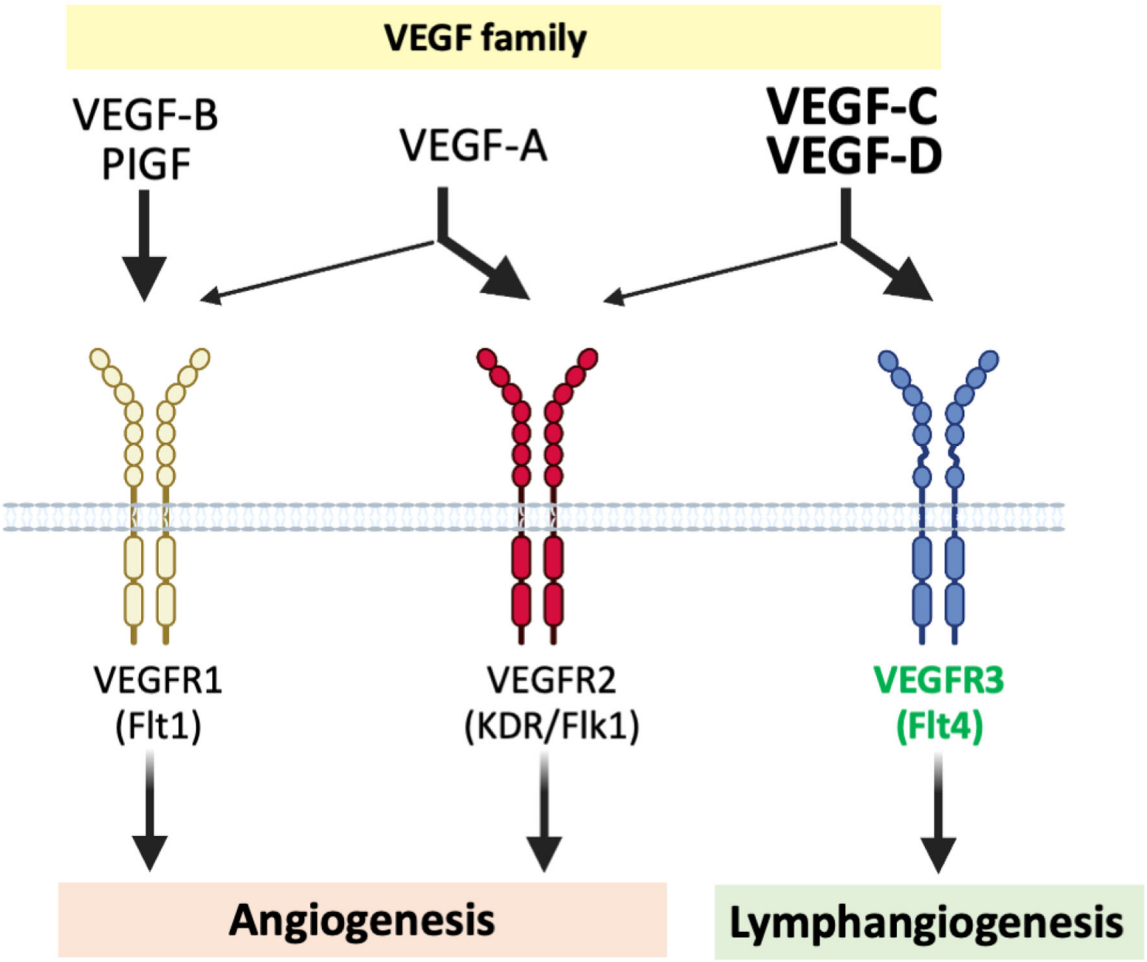

Figure 3. VEGFs and VEGFRs in angiogenesis and lymphangiogenesis

VEGF (Vascular endothelial growth factor) is a potent mediator of both angiogenesis and lymphangiogenesis. All members of the VEGF family induce cellular responses by binding to specific VEGF receptors with tyrosine kinases, leading them to dimerize and activate through phosphorylation. VEGF-C and D bind strongly to VEGFR3/Flt4 and induce lymphangiogenesis, while they also bind very weakly to VEGFR2/KDR. VEGF-A binds to VEGFR1/Flt1 and VEGFR2/KDR and mediates angiogenesis. VEGF-B and placental growth factor (PIGF) bind only to VEGFR1/Flt1. Thick and thin arrows indicate strong and weak binding, respectively. Created with BioRender.com.

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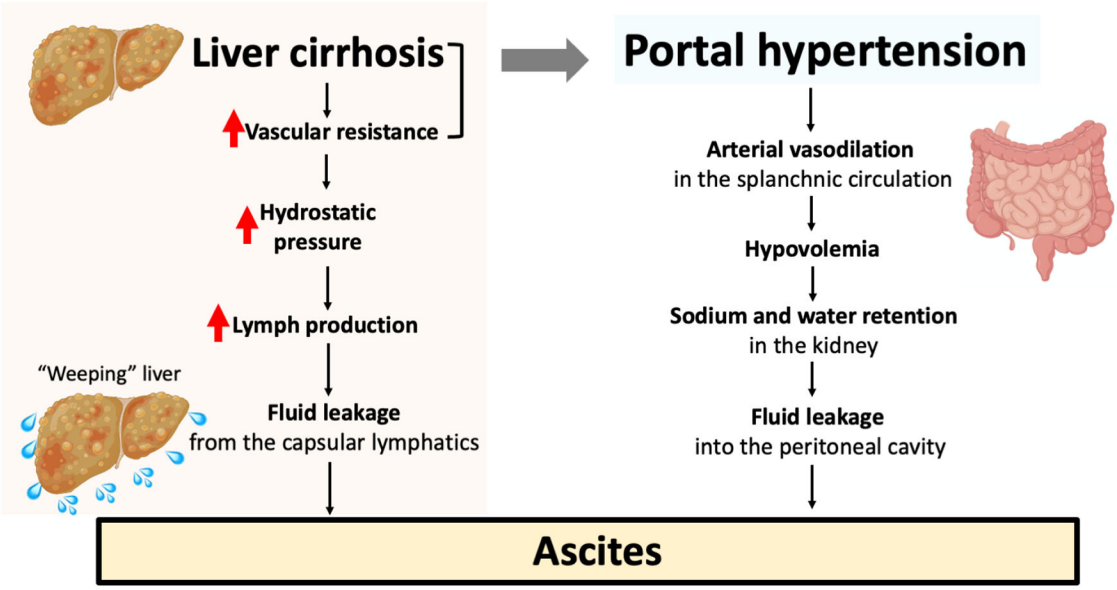

Figure 4. Mechanisms of ascites formation in liver cirrhosis with portal hypertension

Elevated hydrostatic pressure in the sinusoids due to liver cirrhosis causes an increased production of lymph. It is thought that ascitic fluid starts to accumulate when capsular or superficial lymphatics of the liver rupture and hepatic lymph with a high protein concentration leaks into the peritoneal cavity. This lymph leakage from the surface of the liver is known as the so-called 'weeping liver'. When the total fluid flux into the peritoneal cavity exceeds the lymph draining capacity of the peritoneum, ascites forms. According to the "forward theory", portal hypertension causes excessive arterial vasodilation in the splanchnic arterial circulation, leading to the underfilling of the arterial circulation or hypovolemia. Splanchnic arterial vasodilation makes sodium and water retention persistent and leads to leaking fluid into the peritoneal cavity and accumulation of ascites. Created with BioRender.com.